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Dear Sir:

Submitted herewith is the Verified English Translation of French patent application no. 96 09 403, filed on July 19, 1996, to which the above-captioned application claims priority.

No fee is believed to be due for entry and consideration of this paper, however, any required fee may be charged to Deposit Account No. 50-0320.

Respectfully submitted,

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Sir :

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That my name and post office address are as stated below, and
That I am knowledgeable in the English and French languages, that the attached English translation is a true and complete translation of the French patent application No. 96 09 403 filed July 19, 1996 which serves as a priority basis for the United States Patent Application Serial No. 10/085,519.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true ; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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POLYNUCLEOTIDE VACCINE FORMULA IN PARTICULAR AGAINST BOVINE RESPIRATORY PATHOLOGY

The present invention relates to a vaccine formula allowing the vaccination of bovines in particular against respiratory pathology. It also relates to a corresponding method of vaccination.

All bovines are carriers of viruses and bacteria which are potentially pathogenic in widely variable degrees.

Viruses can multiply when the specific immunity is weakened and when there are lesions of the respiratory tract. They are then excreted by the animal and may then contaminate other animals.

Among the viruses which are encountered, there may be mentioned in particular the type 3 parainfluenza virus (PI-3), of moderate inherent pathogenicity, the bovine respiratory syncytial virus (RSV) and the bovine herpesvirus (BHV) also called infectious bovine rhinotracheitis (IBR) virus, of high inherent pathogenicities.

Another virus which is particularly important for its immunodepressant role and its harmful effects on reproduction is the mucosal disease virus or bovine pestivirus (BVDV).

These viruses generally manifest themselves by a primary phase of hyperthermia, flu syndrome and respiratory disorders, with digestive disorders (diarrhoeas) in the case of BVD. This phase may be accompanied by a secondary phase with the onset of bronchopneumonia linked to bacterial, in particular Pasteurella, infections which can lead to death. This phenomenon is exacerbated in particular by the immunodepression resulting from BVD infection or by the infection of macrophages by PI-3. Other symptoms may further appear, such as abortions with BVD and BHV.

It therefore appears necessary to try to develop an effective prevention against the principal

viruses involved in bovine respiratory pathology.

Associations of vaccines against certain viruses responsible for bovine respiratory pathology have already been proposed in the past.

The associations developed so far were prepared from inactivated vaccines or live vaccines and, optionally, mixtures of such vaccines. Their development poses problems of compatibility between valencies and of stability. It is indeed necessary to ensure both the compatibility between the different vaccine valencies, whether from the point of view of the different antigens used or from the point of view of the formulations themselves, especially in the case where both inactivated vaccines and live vaccines are combined. The problem of the conservation of such combined vaccines and also of their safety especially in the presence of an adjuvant also exists. These vaccines are in general quite expensive.

Patent Applications WO-A-90 11092, WO-A-93 19183, WO-A-94 21797 and WO-A-95 20660 have made use of the recently developed technique of polynucleotide vaccines. It is known that these vaccines use a plasmid capable of expressing, in the host's cells, the antigen inserted into the plasmid. All the routes of administration have been proposed (intraperitoneal, intravenous, intramuscular, transcutaneous, intradermal, mucosal and the like). Various vaccination means can also be used, such as DNA deposited at the surface of gold particles and projected so as to penetrate into the animal's skin (Tang et al., Nature 356, 152-154, 1992) and liquid jet injectors which make it possible to transfect at the same time the skin, the muscle, the fatty tissues and the mammary tissues (Furth et al., Analytical Biochemistry, 205, 365-368, 1992).

The polynucleotide vaccines may also use both naked DNAs and DNAs formulated, for example, inside cationic lipid or liposomes.

G.J.M. COX has already proposed polynucleotide

vaccination against type 1 bovine herpes virus in J. of Virology, Volume 67, No. 9, September 1993, 5664-5667. The authors have also described plasmids integrating the gI (gB), gIII (gC) and gIV (gD) genes.

In Vaccine, Volume 13, No. 4, 415-421, 1995, J.E. CROWE presents a general review of the different methods of vaccination against respiratory syncytial virus and against type 3 parainfluenza virus. This review reexamines all the possibilities offered by the current vaccination techniques and simply suggests that the polynucleotide immunization technique could be useful in the immunization strategy against RSV and PI-3. No plasmid construction or result of vaccination of bovines against these viruses is described in this document.

The invention therefore proposes to provide a multivalent vaccine formula which makes it possible to ensure vaccination against a number of pathogenic viruses involved in particular in bovine respiratory pathology and thus to ensure effective vaccination against this pathology.

Another objective of the invention is to provide such a vaccine formula combining different valencies while exhibiting all the criteria required for mutual compatibility and stability of the valencies.

Another objective of the invention is to provide such a vaccine formula which makes it possible to combine different valencies in the same vehicle.

Another objective of the invention is to provide such a vaccine formula which is easy and inexpensive to use.

Yet another objective of the invention is to provide such a vaccine formula and a method for vaccinating bovines which make it possible to obtain a multivalent protection with a high level of efficiency and of long duration, as well as good safety and an absence of residues.

The subject of the present invention is there-

fore a vaccine formula in particular against bovine respiratory pathology, comprising at least three polynucleotide vaccine valencies each comprising a plasmid integrating, so as to express it in vivo in the host cells, a gene with one bovine respiratory pathogen valency, these valencies being selected from the group consisting of bovine herpesvirus, bovine respiratory syncytial virus, mucosal disease virus and type 3 parainfluenza virus, the plasmids comprising, for each valency, one or more of the genes selected from the group consisting of gB and gD for the bovine herpesvirus, F and G for the bovine respiratory syncytial virus, E2, C + E1 + E2 and E1 + E2 for the mucosal disease virus, and HN and F for the type 3 parainfluenza virus.

Valency in the present invention is understood to mean at least one antigen providing protection against the virus for the pathogen considered, it being possible for the valency to contain, as subvalency, one or more modified or natural genes from one or more strains of the pathogen considered.

Pathogenic agent gene is understood to mean not only the complete gene but also the various nucleotide sequences, including fragments which retain the capacity to induce a protective response. The notion of a gene covers the nucleotide sequences equivalent to those described precisely in the examples, that is to say the sequences which are different but which encode the same protein. It also covers the nucleotide sequences of other strains of the pathogen considered, which provide cross-protection or a protection specific for a strain or for a strain group. It also covers the nucleotide sequences which have been modified in order to facilitate the in vivo expression by the host animal but encoding the same protein.

Preferably, the vaccine formula according to the invention comprises the four valences.

As regards the BHV valency, use is preferably made of the two genes encoding gB and gD, in different

plasmids or in one and the same plasmid. Optionally, but less preferably, either of these genes can be used.

For the RSV valency, use is preferably made of the two G and F genes integrated into two different plasmids or into one and the same plasmid. Optionally, but less preferably, the F gene can be used alone.

For the BVD valency, use will preferably be made of a plasmid integrating the E2 gene. Optionally, but less preferably, a plasmid coding for E1 and E2 together or for the combination consisting of C, E1 and E2 can be used.

For the PI-3 valency, use is preferably made of the combination of the two HN and F genes in two different plasmids or in one and the same plasmid. It is also possible to use only the HN gene.

A preferred vaccine formula according to the invention comprises and ensures the expression of the BHV gB and gD genes, the RSV G and F genes, the BVD E2 genes and PI-3 HN and F genes.

The vaccine formula according to the invention can be provided in a dose volume of between 0.1 and 10 ml and in particular between 1 and 5 ml.

The dose will be generally between 10 ng and 1 mg, preferably between 100 ng and 500 μg and more preferably between 1 μg and 250 μg per plasmid type.

Use will preferably be made of naked plasmids simply placed in the vaccination vehicle which will be in general saline (0.9% NaCl), ultrapure water, TE buffer and the like. All the polynucleotide vaccine forms described in the prior art can of course be used.

Each plasmid comprises a promoter capable of ensuring the expression of the gene inserted, under its control, into the host cells. This will be in general a strong eukaryotic promoter and in particular a cytomegalovirus early CMV-IE promoter of human or murine origin, or optionally of another origin such as rats, pigs and guinea pigs.

More generally, the promoter may be either of

viral origin or of cellular origin. As viral promoter other than CMV-IE, there may be mentioned the SV40 virus early or late promoter or the Rous sarcoma virus LTR promoter. It may also be a promoter from the virus from which the gene is derived, for example the gene's own promoter.

As cellular promoter, there may be mentioned the promoter of a cytoskeleton gene, such as for example the desmin promoter (Bolmont et al., Journal of Submicroscopic Cytology and Pathology, 1990, 22, 117-122; and Zhenlin et al., Gene, 1989, 78, 243-254), or alternatively the actin promoter.

When several genes are present in the same plasmid, these may be presented in the same transcription unit or in two different units.

The combination of the different vaccine valencies according to the invention may be preferably achieved by mixing the polynucleotide plasmids expressing the antigen(s) of each valency, but it is also possible to envisage causing antigens of several valencies to be expressed by the same plasmid.

The subject of the invention is also monovalent vaccine formulae comprising one or more plasmids encoding one or more genes from one of the viruses selected from the group consisting of BRSV, BVD and PI-3, the genes being those described above. Besides their monovalent character, these formulae may possess the characteristics stated above as regards the choice of the genes, their combinations, the composition of the plasmids, the dose volumes, the doses and the like.

The monovalent vaccine formulae may be used (i) for the preparation of a polyvalent vaccine formula as described above, (ii) individually against the actual pathology, (iii) combined with a vaccine of another type (live or inactivated whole, recombinant, subunit) against another pathology, or (iv) as booster for a vaccine as described below.

The subject of the present invention is in fact also the use of one or more plasmids according to the

invention for the manufacture of a vaccine intended to vaccinate bovines first vaccinated by means of a first conventional vaccine of the type in the prior art, in particular, selected from the group consisting of a live whole vaccine, an inactivated whole vaccine, a subunit vaccine, a recombinant vaccine, this first vaccine having, that is to say containing or capable of expressing, the antigen(s) encoded by the **plasmids** or antigen(s) providing cross-protection.

Remarkably, the polynucleotide vaccine has a potent booster effect which results in an amplification of the immune response and the acquisition of a long-lasting immunity.

In general, the first-vaccination vaccines can be selected from commercial vaccines available from various veterinary vaccine producers.

The subject of the invention is also a vaccination kit grouping together a first-vaccination vaccine as described above and a vaccine formula according to the invention for the booster. It also relates to a vaccine formula according to the invention accompanied by a leaflet indicating the use of this formula as a booster for a first vaccination as described above.

The subject of the present invention is also a method for vaccinating bovines against respiratory pathology, comprising the administration of the effective vaccine formula as described above. This vaccination method comprises the administration of one or more doses of the vaccine formula, it being possible for these doses to be administered in succession over a short period of time and/or in succession at widely spaced intervals.

The vaccine formulae according to the invention can be administered, in the context of this method of vaccination, by the different routes of administration proposed in the prior art for polynucleotide vaccination and by means of known techniques of administration.

The subject of the invention is also the method

of vaccination consisting in making a first vaccination as described above and a booster with a vaccine formula according to the invention.

In a preferred embodiment of the process according to the invention, there is administered in a first instance, to the animal, an effective dose of the conventional type vaccine especially inactivated, live, attenuated or recombinant, type, or alternatively a subunit vaccine, so as to provide a first vaccination, and, within a period preferably of 2 to 6 weeks, the polyvalent or monovalent vaccine according to the invention is administered.

The invention also relates to the method of preparing the vaccine formulae, namely the preparation of the valencies and mixtures thereof, as evident from this description.

The invention will now be described in greater detail with the aid of the embodiments of the invention taken with reference to the accompanying drawings.

List of figures

Sequence listing SEQ ID No.

SEQ ID No. 1	:	Sequence of the BHV-1 gB gene (strain ST)
SEQ ID No. 2	:	Oligonucleotide PB234
SEQ ID No. 3	:	Oligonucleotide PB235
SEQ ID No. 4	:	Oligonucleotide AB162

SEQ ID No. 5	:	Oligonucleotide AB163
SEQ ID No. 6	:	Oligonucleotide AB026
SEQ ID No. 7	:	Oligonucleotide AB027
SEQ ID No. 8	:	Oligonucleotide AB028
SEQ ID No. 9	:	Oligonucleotide AB029
SEQ ID No. 10	:	Oligonucleotide AB110
SEQ ID No. 11	:	Oligonucleotide AB111
SEQ ID No. 12	:	Oligonucleotide AB114
SEQ ID No. 13	:	Oligonucleotide AB115
SEQ ID No. 14	:	Oligonucleotide AB116
SEQ ID No. 15	:	Oligonucleotide AB117
SEQ ID No. 16	:	Oligonucleotide AB130
SEQ ID No. 17	:	Oligonucleotide AB131
SEQ ID No. 18	:	Oligonucleotide AB132
SEQ ID No. 19	:	Oligonucleotide AB133

EXAMPLES

Example 1: Culture of the viruses

The viruses are cultured on the appropriate cellular system until a cytopathic effect is obtained. The cellular systems to be used for each virus are well known to persons skilled in the art. Briefly, the cells sensitive to the virus used, which are cultured in Eagle's minimum essential medium (MEM medium) or another appropriate medium, are inoculated with the viral strain studied using a multiplicity of infection of 1. The infected cells are then incubated at 37°C for the time necessary for the appearance of a complete cytopathic effect (on average 36 hours).

Example 2: Extraction of the viral genomic DNAs

After culturing, the supernatant and the lysed cells are harvested and the entire viral suspension is centrifuged at 1000 g for 10 minutes at +4°C so as to remove the cellular debris. The viral particles are then harvested by ultracentrifugation at 400,000 g for 1 hour at +4°C. The pellet is taken up in a minimum volume of buffer (10 mM Tris, 1 mM EDTA). This concentrated viral

suspension is treated with proteinase K (100 μg/ml final) in the presence of sodium dodecyl sulphate (SDS) (0.5% final) for 2 hours at 37°C. The viral DNA is then extracted with a phenol/chloroform mixture and then precipitated with 2 volumes of absolute ethanol. After leaving overnight at -20°C, the DNA is centrifuged at 10,000 g for 15 minutes at +4°C. The DNA pellet is dried and then taken up in a minimum volume of sterile ultrapure water. It can then be digested with restriction enzymes.

Example 3: Isolation of the viral genomic RNAs

The RNA viruses were purified according to techniques well known to persons skilled in the art. The genomic viral RNA of each virus was then isolated using the "guanidium thiocyanate/phenol-chloroform" extraction technique described by P. Chomczynski and N. Sacchi (Anal. Biochem., 1987, **162**, 156-159).

Example 4: Molecular biology techniques

All the constructions of plasmids were carried out using the standard molecular biology techniques described by J. Sambrook *et al.* *(Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989). All the restriction fragments used for the present invention were isolated using the "Geneclean" kit (BIO 101 Inc. La Jolla, CA).

Example 5: RT-PCR technique

Specific oligonucleotides (comprising restriction sites at their 5' ends to facilitate the cloning of the amplified fragments) were synthesized such that they completely cover the coding regions of the genes which are to be amplified (see specific examples). The reverse transcription (RT) reaction and the polymerase chain reaction (PCR) were carried out according to standard techniques (J. Sambrook *et al., Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor

Laboratory, Cold Spring Harbor, New York, 1989). Each RT-PCR reaction was performed with a pair of specific amplimers and taking, as template, the viral genomic RNA extracted. The complementary DNA amplified was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) before being digested with restriction enzymes.

Example 6: plasmid pVR1012

The plasmid pVR1012 (Figure No. 1) was obtained from Vical Inc., San Diego, CA, USA. Its construction has been described in J. Hartikka *et al.* (Human Gene Therapy, 1996, **7**, 1205-1217).

Example 7: Construction of the plasmid pPB156 (BHV-1 gB gene)

The BVH-1 bovine herpesvirus (ST strain) genomic DNA (Leung-Tack P. *et al.* Virology, 1994, **199**, 409-421) was prepared according to the technique described in Example 2 and was digested with BamHI. After purification, the 18 kbp BamHI-BamHI fragment was cloned into the vector pBR322, previously digested with BamH1, to give the plasmid pIBR-4-BamHI (22 kbp).

The plasmid pIBR-4-BamHI was then digested with SalI in order to liberate a 6.6 kbp SalI-SalI fragment containing the gene encoding the BHV-1 gB glycoprotein (Figure No. 2 and SEQ ID No. 1). This fragment was cloned into the vector pBR322, previously digested with SalI, to give the plasmid pIBR-6,6-SalI (10.9 kbp).

The plasmid pIBR-6,6-SalI was digested with NheI and BglII in order to liberate a 2676 bp NheI-BglII fragment containing the gene encoding the bovine herpesvirus (BHV-1) gB glycoprotein (fragment A).

A PCR reaction was carried out with the genomic DNA from the bovine herpesvirus (BHV-1) (ST strain) and with the following oligonucleotides:

PB234 (30 mer) (SEQ ID No. 2)

5'TTGTCGACATGGCCGCTCGCGGCGGTGCTG 3'

PB235 (21 mer) (SEQ ID No. 3)

5'GCAGGGCAGCGGCTAGCGCGG 3'

so as to isolate the 5′ part of the gene encoding the BHV-1 gB glycoprotein. After purification, the 153 bp PCR product was digested with SalI and NheI in order to isolate a 145 bp SalI-NheI fragment (fragment B).

The fragments A and B were ligated together with the vector pVR1012 (Example 6), previously digested with SalI and BamHI, to give the plasmid pPB156 (7691 bp) (Figure No. 3).

Example 8: Construction of the plasmid pAB087 (BHV-1 gD gene)

A PCR reaction was carried out with the genomic DNA from the bovine herpesvirus (BHV-1) (ST strain) (P. Leung-Tack *et al.*, Virology, 1994, **199**, 409-421), prepared according to the technique described in Example 2, and with the following oligonucleotides:

AB162 (31 mer) (SEQ ID No. 4)

5′AAACTGCAGATGCAAGGGCCGACATTGGCCG 3′

AB163 (30 mer) (SEQ ID No. 5)

5′ATCTTGTACCATATGACCGTGGCGTTG 3′

so as to amplify the 5′ part of the gene encoding the bovine herpesvirus (BHV-1) gD glycoprotein (GenBank sequence accession No. = L26360) in the form of a 338 bp PCR fragment. After purification, this fragment was digested with PstI and NdeI in order to isolate a 317 bp PstI-NdeI fragment (fragment A).

The plasmid pBHV001 (P. Leung-Tack *et al.*, Virology, 1994, **199**, 409-421) was digested with NdeI and StyI in order to liberate a 942 bp fragment containing the 3′ part of the gene encoding the BHV-1 gD glycoprotein (fragment B).

The fragments A and B were ligated together with the vector pVR1012 (Example 6), previously digested with PstI and XbaI, to give the plasmid pAB087 (6134 bp) (Figure No. 4).

Example 9: Construction of the plasmid pAB011 (BRSV F gene)

An RT-PCR reaction according to the technique

described in Example 5 was carried out with the genomic RNA from the bovine respiratory syncytial virus (BRSV) (391-2 strain) (R. Lerch *et al.*, Virology, 1991, **181**, 118-131), prepared as indicated in Example 3, and with the following oligonucleotides:

AB026 (33 mer) (SEQ ID No. 6)

5'AAAACTGCAGGGATGGCGGCAACAGCCATGAGG 3'

AB027 (31 mer) (SEQ ID No. 7)

5'CGCGGATCCTCATTTACTAAAGGAAAGATTG 3'

so as to isolate the gene encoding the F fusion glycoprotein (BRSV F) in the form of a 1734 bp PCR fragment. After purification, this fragment was digested with PstI and BamHI in order to isolate a 1717 bp PstI-BamHI fragment. This fragment was ligated with the vector pVR1012 (Example 6), previously digested with PstI and BamHI, to give the plasmid pAB011 (6587 bp) (Figure No. 5).

Example 10: Construction of the plasmid pAB012 (BRSV G gene)

An RT-PCR reaction according to the technique described in Example 5 was carried out with the genomic RNA from the bovine respiratory syncytial virus (BRSV) (391-2 strain) (R. Lerch *et al.*, J. Virology, 1990, **64**, 5559-5569) and with the following oligonucleotides:

AB028 (32 mer) (SEQ ID No. 8)

5'AAAACTGCAGATGTCCAACCATACCCATCATC 3'

AB029 (35 mer) (SEQ ID No. 9)

5'CGCGGATCCCTAGATCTGTGTAGTTGATTGATTTG 3'

so as to isolate the gene encoding the G protein (BRSV G) in the form of a 780 bp PCR fragment. After purification, this fragment was digested with PstI and BamHI in order to isolate a 763 bp PstI-BamHI fragment. This fragment was ligated with the vector pVR1012 (Example 6), previously digested with PstI and BamHI, to give the plasmid pAB012 (5634 bp) (Figure No. 6).

Example 11: Construction of the plasmid pAB058 (BVDV C gene)

An RT-PCR reaction according to the technique described in Example 5 was carried out with the genomic RNA from the bovine viral diarrhoea virus (BVDV) (Osloss strain) (L. De Moerlooze *et al.*, J. Gen. Virol., 1993, **74**, 1433-1438), prepared according to the technique described in Example 3, and with the following oligonucleotides:

AB110 (35 mer) (SEQ ID No. 10)

5'AAAACTGCAGATGTCCGACACAAAAGCAGAAGGGG 3'

AB111 (47 mer) (SEQ ID No. 11)

5'CGCGGATCCTCAATAAAAATCATTCCCACTGCGACTTGAAACAAAAC 3'

so as to amplify a 342 bp fragment containing the gene encoding the C capside protein from the BVDV virus. After purification, the RT-PCR product was digested with PstI and BamHI to give a 324 bp PstI-BamHI fragment.

This fragment was ligated with the vector pVR1012 (Example 6), previously digested with PstI and BamHI, to give the plasmid pAB058 (5183 bp) (Figure No. 7).

Example 12: Construction of the plasmid pAB059 (BVDV E1 "gene")

An RT-PCR reaction according to the technique described in Example 5 was carried out with the genomic RNA from the bovine viral diarrhoea virus (BVDV) (Osloss strain) (L. De Moerlooze *et al.*, J. Gen. Virol., 1993, **74**, 1433-1438) and with the following oligonucleotides:

AB114 (32 mer) (SEQ ID No. 12)

5'ACGCGTCGACATGAAGAAACTAGAGAAAGCCC 3'

AB115 (33 mer) (SEQ ID No. 13)

5' CGCGGATCCTCAGCCGGGTTTGCAAACTGGGAG 3'

so as to isolate the sequence encoding the BVDV virus E1 protein in the form of a 1381 bp PCR fragment. After purification, this fragment was digested with SalI and BamHI to give a 1367 bp SalI-BamHI fragment.

This fragment was ligated with the vector pVR1012 (Example 6), previously digested with SalI and

BamHI, to give the plasmid pAB059 (6236 bp) (Figure No. 8).

Example 13: Construction of the plasmid pAB060 (BVDV E2 "gene")

An RT-PCR reaction according to the technique described in Example 5 was carried out with the genomic RNA from the bovine viral diarrhoea virus (BVDV) (Osloss strain) (L. De Moerlooze *et al.*, J. Gen. Virol., 1993, **74**, 1433-1438) and with the following oligonucleotides:

AB116 (36 mer) (SEQ ID No. 14)

5'ACGCGTCGACATGACGACTACTGCATTCCTGGTATG 3'

AB117 (33 mer) (SEQ ID No. 15)

5'CGCGGATCCTCATTGACGTCCCGAGGTCATTTG 3'

so as to isolate the sequence encoding the BVDV virus E2 protein in the form of a 1252 bp PCR fragment. After purification, this fragment was digested with SalI and BamHI to give a 1238 bp SalI-BamHI fragment.

This fragment was ligated with the vector pVR1012 (Example 6), previously digested with SalI and BamHI, to give the plasmid pAB060 (6107 bp) (Figure No. 9).

Example 14: Construction of the plasmid pAB071 (BPIV HN gene)

An RT-PCR reaction according to the technique described in Example 5 was carried out with the genomic RNA from the type 3 bovine parainfluenza virus (PI3 = BPIV) and with the following oligonucleotides:

AB130 (33 mer) (SEQ ID No. 16)

5'TTTGTCGACATGGAATATTGGAAACACACAAAC 3'

AB131 (33 mer) (SEQ ID No. 17)

5'TTTGGATCCTTAGCTGCAGTTTTTCGGAACTTC 3'

so as to isolate the gene encoding the BPIV HN glycoprotein (HN gene sequence deposited by H. Shibuta in 1987. GenBank sequence accession No. = Y00115) in the form of a 1737 bp PCR fragment. After purification, this fragment was digested with SalI and BamHI in order to isolate a 1725 bp SalI-BamHI fragment. This fragment

was ligated with the vector pVR1012 (Example 6), previously digested with SalI and BamHI, to give the plasmid pAB071 (6593 bp) (Figure No. 10).

Example 15: Construction of the plasmid pAB072 (BPIV F gene)

An RT-PCR reaction according to the technique described in Example 5 was carried out with the genomic RNA from the type 3 bovine parainfluenza virus (PI3 = BPIV) and with the following oligonucleotides:

AB132 (30 mer) (SEQ ID No. 18)

5'TTTGTCGACATGATCATCACAAACACAATC 3'

AB133 (30 mer) (SEQ ID No. 19)

5'TTTGGATCCTCATTGTCTACTTGTTAGTAC 3'

so as to isolate the gene encoding the BPIV F glycoprotein (F gene sequence deposited by H. Shibuta in 1987. GenBank sequence accession No. = Y00115) in the form of a 1641 bp PCR fragment. After purification, this fragment was digested with SalI and BamHI in order to isolate a 1629 bp SalI-BamHI fragment. This fragment was ligated with the vector pVR1012 (Example 6), previously digested with SalI and BamHI, to give the plasmid pAB072 (6497 bp) (Figure No. 11).

Example 16: Preparation and purification of the plasmids

For the preparation of the plasmids intended for the vaccination of animals, any technique may be used which makes it possible to obtain a suspension of purified plasmids predominantly in a supercoiled form. These techniques are well known to persons skilled in the art. There may be mentioned in particular the alkaline lysis technique followed by two successive ultracentrifugations on a caesium chloride gradient in the presence of ethidium bromide as described in J. Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, 2nd edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989). Reference may also be made to Patent Applications PCT WO 95/21250 and PCT WO

96/02658, which describe methods for producing, on an industrial scale, plasmids which can be used for vaccination. For the purposes of the manufacture of vaccines (see Example 17), the purified plasmids are resuspended so as to obtain solutions at a high concentration (> 2 mg/ml) which are compatible with storage. To do this the plasmids are resuspended either in ultrapure water or in TE buffer (10 mM Tris-HCl; 1 mM EDTA, pH 8.0).

Example 17: Manufacture of the associated vaccines

The various plasmids necessary for the manufacture of an associated vaccine are mixed starting with their concentrated solutions (Example 16). The mixtures are prepared such that the final concentration of each plasmid corresponds to the effective dose of each plasmid. The solutions which can be used to adjust the final concentration of the vaccine may be either a 0.9% NaCl solution, or PBS buffer.

Specific formulations such as liposomes or cationic lipids, may also be used for the manufacture of the vaccines.

Example 18: Vaccination of bovines

The bovines are vaccinated with doses of 100 µg, 250 µg or 500 µg per plasmid. The injections are performed with a needle by the intramuscular route either at the level of the *gluteus* muscle, or at the level of the neck muscles. The vaccinal doses are administered in volumes of between 1 and 5 ml.

CLAIMS

1. Bovine vaccine formula against bovine respiratory pathology, comprising at least three polynucleotide vaccine valencies each comprising a plasmid integrating, so as to express it in vivo in the host cells, a gene with one bovine respiratory pathogen valency, these valencies being selected from the group consisting of bovine herpesvirus, bovine respiratory syncytial virus, mucosal disease virus and type 3 parainfluenza virus, the plasmids comprising, for each valency, one or more of the genes selected from the group consisting of gB and gD for the bovine herpesvirus, F and G for the bovine respiratory syncytial virus, E2, C + E1 + E2 and E1 + E2 for the mucosal disease virus, HN and F for the type 3 parainfluenza virus.

2. Vaccine formula according to Claim 1, which comprises the four polynucleotide vaccine valencies.

3. Vaccine formula according to Claim 1 or 2, which comprises the bovine herpesvirus gB and gD genes, in the same plasmid or in different plasmids.

4. Formula according to Claim 1 or 2, which comprises the bovine respiratory syncytial virus F and G genes, in the same plasmid or in different plasmids.

5. Vaccine formula according to Claim 1 or 2, wherein the plasmid for the mucosal disease virus comprises the E2 gene.

6. Vaccine formula according to Claim 1 or 2, wherein, for the type 3 parainfluenza virus valency, it comprises the HN gene in one plasmid or all the genes encoding HN and F in the same plasmid or in different plasmids.

7. Vaccine formula according to all of Claims 1 to 6.

8. Vaccine formula according to any one of Claims 1 to 7, which comprises from 10 ng to 1 mg, preferably from 100 ng to 500 µg, still more preferably from 1 µg to 250 µg of each plasmid.

9. Use of one or more plasmids as described in any one of Claims 1 to 8, for the manufacture of a vaccine intended to vaccinate bovines first vaccinated by means of a first vaccine selected from the group consisting of a live whole vaccine, an inactivated whole vaccine, a subunit vaccine, a recombinant vaccine, this first vaccine having the antigen(s) encoded by the plasmid(s) or antigen(s) providing cross-protection.

10. Vaccination kit grouping together a vaccine formula according to any one of Claims 1 to 8, and a vaccine selected from the group consisting of a live whole vaccine, an inactivated whole vaccine, a subunit vaccine, a recombinant vaccine, this first vaccine having the antigen encoded by the polynucleotide vaccine or an antigen providing cross-protection, for an administration of the latter in first vaccination and as a booster with the vaccine formula.

11. Vaccine formula according to any one of Claims 1 to 8, accompanied by a leaflet indicating that this formula can be used as a booster for a first vaccine selected from the group consisting of a live whole vaccine, an inactivated whole vaccine, a subunit vaccine, a recombinant vaccine, this first vaccine having the antigen encoded by the polynucleotide vaccine or an antigen providing cross-protection.

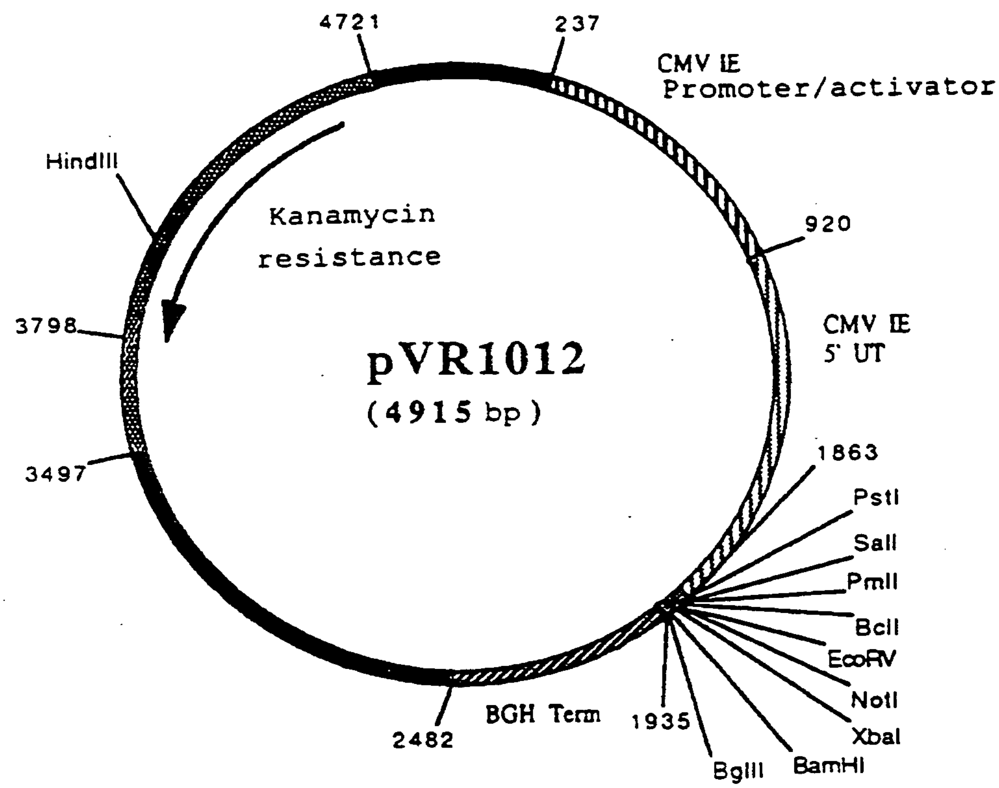
COMPANY CALLED: RHONE MERIEUX

POLYNUCLEOTIDE VACCINE FORMULA IN PARTICULAR AGAINST BOVINE RESPIRATORY PATHOLOGY

ABSTRACT OF THE CONTENT OF THE INVENTION

The bovine vaccine formula against bovine respiratory pathology comprises at least three polynucleotide vaccine valencies each comprising a plasmid integrating, so as to express it in vivo in the host cells, a gene with one bovine respiratory pathogen valency, these valencies being selected from those of the group consisting of bovine herpesvirus, bovine respiratory syncytial virus, mucosal disease virus and type 3 parainfluenza virus, the plasmids comprising, for each valency, one or more of the genes selected from the group consisting of gB and gD for the bovine herpesvirus, F and G for the bovine respiratory syncytial virus, E2, C + E1 + E2 and E1 + E2 for the mucosal disease virus, and HN and F for the type 3 parainfluenza virus.

Figure 4.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237
CMV IE
Promoter/activator
HindIII
Kanamycin
resistance
920
CMV IE
5' UT
3798
pVR1012
(4915 bp)
3497
1863
PstI
SalI
PmlI
BclI
EcoRV
NotI
XbaI
BamHI
BglII
1935
BGH Term
2482

Figure No. 1

```
   1  ATGGCCGCTCGCGGCGGTGCTGAACGCGCCGCGGGCGCCGGAGACGGTCGGCGAGGACAGCGT
   1▶ MetAlaAlaArgGlyGlyAlaGluArgAlaAlaGlyAlaGlyAspGlyArgArgGlyGlnArg

  64  CGTCATCTACGACCGGGACGTGTTCTCGCTGCTCTACGCGGTCCTGCAGCGCCTGGCGCCGGC
  22▶ ArgHisLeuArgProGlyArgValLeuAlaAlaLeuArgGlyProAlaAlaProGlyAlaGly

 127  GGGGCGCGCCGCGCTAGCCGCTGCCCTGCTATGGGCGACGTGGGCCCTGCTGCTGGCGGCG
  43▶ GlyAlaArgAlaAlaLeuAlaAlaAlaLeuLeuTrpAlaThrTrpAlaLeuLeuLeuAlaAla

 190  CCCGCCGCGGGGCGACCGGCGACAACGCCCCCGGCGCCCCCGCCCGAAGAGGCCGCGAGCCCG
  64▶ ProAlaAlaGlyArgProAlaThrThrProProAlaProProProGluGluAlaAlaSerPro

 253  GCGCCCCCCGCGAGCCCCAGCCCCCCCGGCCCCGACGGCGACGACGCCGCCAGCCCCGACAAC
  85▶ AlaProProAlaSerProSerProProGlyProAspGlyAspAspAlaAlaSerProAspAsn

 316  AGCACAGACGTGCGCGCCGCGCTCCGGCTCGCGCAGGCGGCCGGGGAAAACTCGCGCTTCTTC
 106▶ SerThrAspValArgAlaAlaLeuArgLeuAlaGlnAlaAlaGlyGluAsnSerArgPhePhe

 379  GTGTGCCCGCCGCCCTCGGGCGCCACGGTGGTCCGGCTCGCGCCCGCGCGGCCGTGCCCTGAG
 127▶ ValCysProProProSerGlyAlaThrValValArgLeuAlaProAlaArgProCysProGlu

 442  TACGGGCTCGGGCGGAACTACACGGAGGGCATCGGCGTCATTTACAAGGAGAACATCGCGCCG
 148▶ TyrGlyLeuGlyArgAsnTyrThrGluGlyIleGlyValIleTyrLysGluAsnIleAlaPro

 505  TACACGTTCAAGGCCTACATTTACAAAAACGTGATCGTGACCACGACCTGGGCGGGCAGCACG
 169▶ TyrThrPheLysAlaTyrIleTyrLysAsnValIleValThrThrThrTrpAlaGlySerThr

 568  TACGCGGCCATTACAAACCAGTACACGGACCGCGTGCCCGTGGGCATGGGCGAGATCACGGAC
 190▶ TyrAlaAlaIleThrAsnGlnTyrThrAspArgValProValGlyMetGlyGluIleThrAsp

 631  CTGGTGGACAAGAAGTGGCGCTGCCTTTCGAAAGCCGAGTACCTGCGCAGCGGGCGCAAGGTG
 211▶ LeuValAspLysLysTrpArgCysLeuSerLysAlaGluTyrLeuArgSerGlyArgLysVal

 694  GTGGCCTTTGACCGCGACGACGACCCCTGGGAGGCGCCGCTGAAGCCTGCGCGGCTGAGCGCG
 232▶ ValAlaPheAspArgAspAspAspProTrpGluAlaProLeuLysProAlaArgLeuSerAla

 757  CCCGGGGTGCGGGGCTGGCACACGACGGACGATGTGTACACGGCGCTGGGCTCGGCGGGGCTC
 253▶ ProGlyValArgGlyTrpHisThrThrAspAspValTyrThrAlaLeuGlySerAlaGlyLeu

 820  TACCGCACGGGCACCTCTGTGAACTGCATCGTGGAAGAAGTGGAGGCGCGCTCGGTGTACCCG
 274▶ TyrArgThrGlyThrSerValAsnCysIleValGluGluValGluAlaArgSerValTyrPro

 883  TACGACTCGTTCGCGCTCTCGACCGGGGACATTATCTACATGTCGCCCTTTTACGGGCTGCGC
 295▶ TyrAspSerPheAlaLeuSerThrGlyAspIleIleTyrMetSerProPheTyrGlyLeuArg

 946  GAGGGCGCGCACCGCGAGCACACCAGGCTACTCGCCGGAGCGCTTCCAGCAGATCGAGGGCTA
 316▶ GluGlyAlaHisArgGluHisThrArgLeuLeuAlaGlyAlaLeuProAlaAspArgGlyLeu

1009  CTACAAGCGCGACATGGCCACGGGCCGGCGCCTCAAGGAGCCGGTCTCGCGGAACTTTTTGCG
 337▶ LeuGlnAlaArgHisGlyHisGlyProAlaProGlnGlyAlaGlyLeuAlaGluLeuPheAla

1072  TACACAGCACGTGACGGTAGCCTGGGACTGGGTGCCCAAGCGCAAAAACGTGTGCTCGCTGGC
 358▶ TyrThrAlaArgAspGlySerLeuGlyLeuGlyAlaGlnAlaGlnLysArgValLeuAlaGly
```

Figure No. 2

1135 CAAGTGGCGCGAGGCGGACGAAATGCTGCGAGACGAGAGCCGCGGGAACTTCCGCTTCACGGC
379▸ GlnValAlaArgGlyGlyArgAsnAlaAlaArgArgGluProArgGluLeuProLeuHisGly

1198 CCGCTCGCTCTCGGCGACCTTTGTGAGCGACAGCCACACCTTCGCGTTGCAGAATGTGCCGCT
400▸ ProLeuAlaLeuGlyAspLeuCysGluArgGlnProHisLeuArgValAlaGluCysAlaAla

1261 GAGCGACTGCGTGATCGAAGAGGCCGAGGCCGCGGTCGAGCGCGTCTACCGCGAGCGCTACAA
421▸ GluArgLeuArgAspArgArgGlyArgGlyArgGlyArgAlaArgLeuProArgAlaLeuGln

1324 CGGCACGCACGTGCTGTCGGGCAGCTTGGAGACGTACCTGGCGCGCGGCGGCTTTGTCGTGGC
442▸ ArgHisAlaArgAlaValGlyGlnLeuGlyAspValProGlyAlaArgArgLeuCysArgGly

1387 CTTCCGGCGATGCTCAGCAACGAGCTGGCCAAGCTGTACCTGCAGGAGCTGGCGCGCTCGAAC
463▸ LeuProAlaMetLeuSerAsnGluLeuAlaLysLeuTyrLeuGlnGluLeuAlaArgSerAsn

1450 GGCACGCTCGAGGGGCTGTTCGCCGCCGCGGCGCCCAAGCCGGGCCCGCGGCGCGCGCGCCGC
484▸ GlyThrLeuGluGlyLeuPheAlaAlaAlaAlaProLysProGlyProArgArgAlaArgArg

1513 GCCGCGCCGTCTGCGCCCGGCGGCCCGGGCGCGGCCAACGGGCCCGCCGGCGACGGCGACGCC
505▸ AlaAlaProSerAlaProGlyGlyProGlyAlaAlaAsnGlyProAlaGlyAspGlyAspAla

1576 GGCGGGCGGGTGACTACCGTGAGCTCGGCCGAGTTTGCGGCGCTGCAGTTCACCTACGACCAC
526▸ GlyGlyArgValThrThrValSerSerAlaGluPheAlaAlaLeuGlnPheThrTyrAspHis

1639 ATCCAGGACCACGTGAACACCATGTTCAGCCGCCTGGCCACGTCCTGGTGCCTGCTGCAGAAC
547▸ IleGlnAspHisValAsnThrMetPheSerArgLeuAlaThrSerTrpCysLeuLeuGlnAsn

1702 AAGGAGCGCGCCCTGTGGGCCGAGGCGGCTAAGCTCAACCCCAGCGCGGCGGCCAGCGCTGCG
568▸ LysGluArgAlaLeuTrpAlaGluAlaAlaLysLeuAsnProSerAlaAlaAlaSerAlaAla

1765 CTGGACCGCCGCGCCGCCGCGCGCATGTTGGGGGACGCCATGGCCGTGACGTACTGCCACGAG
589▸ LeuAspArgArgAlaAlaAlaArgMetLeuGlyAspAlaMetAlaValThrTyrCysHisGlu

1828 CTGGGCGAGGGGCGCGTGTTCATCGAGAACTCGATGCGCGCGCCCGGCGGCGTTTGCTACAGC
610▸ LeuGlyGluGlyArgValPheIleGluAsnSerMetArgAlaProGlyGlyValCysTyrSer

1891 CGCCCGCCGGTCTCCTTTGCCTTCGGCAACGAGAGCGAGCCGGTGGAGGGCCAGCTCGGCGAG
631▸ ArgProProValSerPheAlaPheGlyAsnGluSerGluProValGluGlyGlnLeuGlyGlu

1954 GACAACGAGCTGCTGCCGGGCCGCGAGCTCGTGGAGCCCTGCACCGCCAACCACAAGCGCTAC
652▸ AspAsnGluLeuLeuProGlyArgGluLeuValGluProCysThrAlaAsnHisLysArgTyr

2017 TTCCGCTTTGGCGCGGACTACGTGTACTACGAGAACTACGCGTACGTGCGGCGGGTCCCGCTC
673▸ PheArgPheGlyAlaAspTyrValTyrTyrGluAsnTyrAlaTyrValArgArgValProLeu

2080 GCGGAGCTGGAGGTGATCAGCACCTTTGTGGACCTAAACCTCACGGTTCTGGAGGACCGCGAG
694▸ AlaGluLeuGluValIleSerThrPheValAspLeuAsnLeuThrValLeuGluAspArgGlu

2143 TTCTTGCCGCTAGAAGTGTACACGCGCGCCGAGCTCGCCGACACGGGTCTGCTCGACTACAGC
715▸ PheLeuProLeuGluValTyrThrArgAlaGluLeuAlaAspThrGlyLeuLeuAspTyrSer

2206 GAGATACAGCGCCGCAACCAGCTGCACGAGCTCCGGTTCTACGACATTGACCGCGTGGTCAAG
736▸ GluIleGlnArgArgAsnGlnLeuHisGluLeuArgPheTyrAspIleAspArgValValLys

Figure No. 2 (continuation)

2269 ACGGACGGCAATATGGCCATCATGCGAGGGCTCGCCAACTTCTTTCAGGGCCTGGGCGCCGTC
757▸ThrAspGlyAsnMetAlaIleMetArgGlyLeuAlaAsnPhePheGlnGlyLeuGlyAlaVal

2332 GGGCAGGCGGTGGGCACGGTGGTGCTGGGCGCCGCGGGTGCCGCGCTCTCGACCGTGTCGGGC
778▸GlyGlnAlaValGlyThrValValLeuGlyAlaAlaGlyAlaAlaLeuSerThrValSerGly

2395 ATCGCCTCGTTTATTGCGAACCCGTTCGGCGCGCTGGCCACGGGGCTGCTGGTGCTCGCCGGG
799▸IleAlaSerPheIleAlaAsnProPheGlyAlaLeuAlaThrGlyLeuLeuValLeuAlaGly

2458 CTGGTGGCCGCTTTCCTGGCGTACCGGTACATTTCCCGCCTCCGCAGCAACCCCATGAAGGCG
820▸LeuValAlaAlaPheLeuAlaTyrArgTyrIleSerArgLeuArgSerAsnProMetLysAla

2521 CTGTACCCGATCACCACGCGCGCGCTCAAGGACGACGCCCGGGGCGCAACCGCCCCGGGCGAG
841▸LeuTyrProIleThrThrArgAlaLeuLysAspAspAlaArgGlyAlaThrAlaProGlyGlu

2584 GAAGAGGAGGAGTTTGACGCGGCCAAACTGGAGCAGGCCCGCGAGATGATCAAGTATATGTCG
862▸GluGluGluGluPheAspAlaAlaLysLeuGluGlnAlaArgGluMetIleLysTyrMetSer

2647 CTCGTGTCAGCGGTCGAGCGGCAAGAGCACAAGGCGAAAAAGAGCAACAAGGGCGGCCCGCTG
883▸LeuValSerAlaValGluArgGlnGluHisLysAlaLysLysSerAsnLysGlyGlyProLeu

2710 CTGGCGACCCGGCTGACGCAGCTCGCGCTTCGGCGGCGAGCGCCGCCGGAGTACCAGCAGCTT
904▸LeuAlaThrArgLeuThrGlnLeuAlaLeuArgArgArgAlaProProGluTyrGlnGlnLeu

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Figure No. 2 (end)

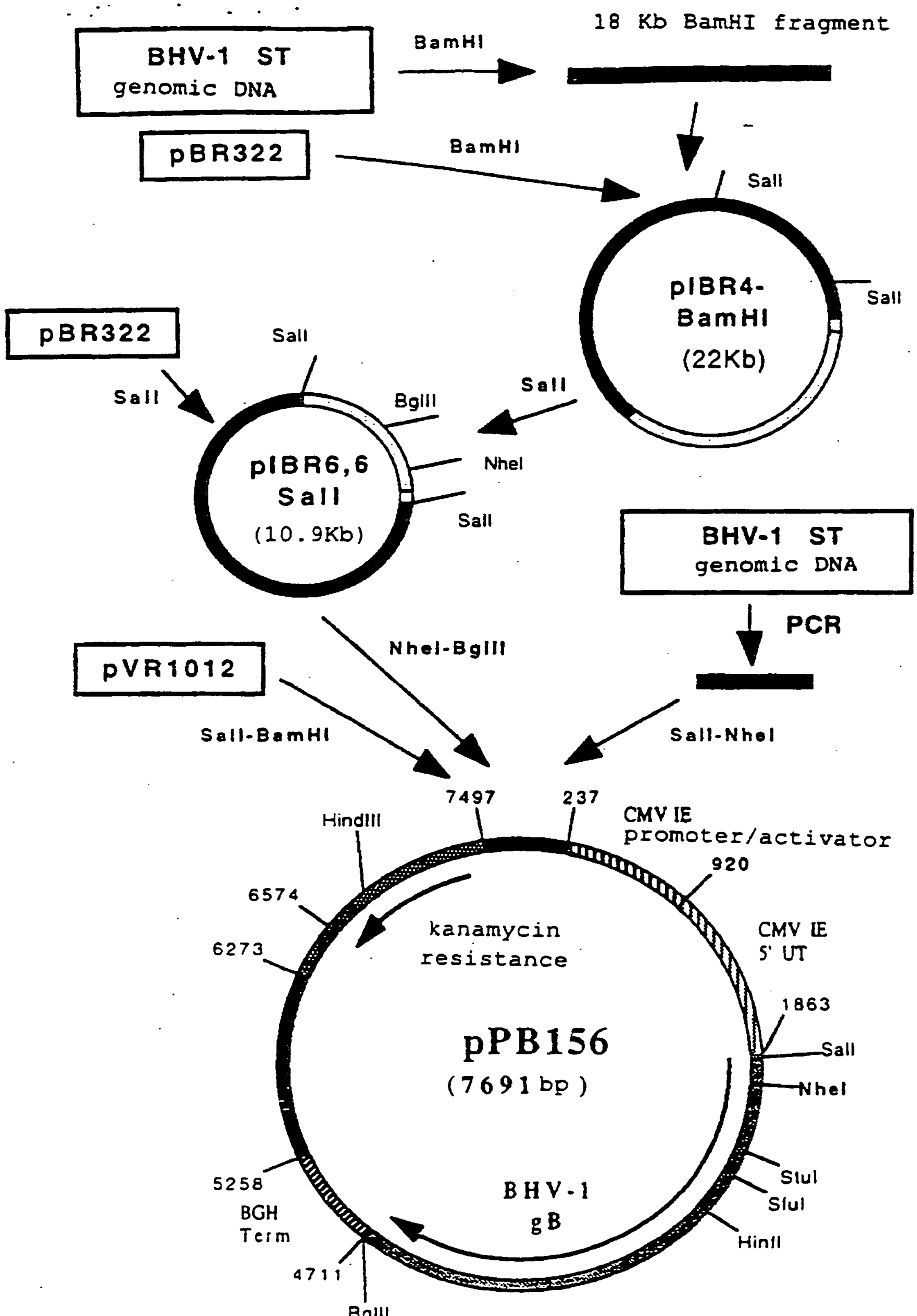

18 Kb BamHI fragment
BHV-1 ST
genomic DNA
BamHI
pBR322
BamHI
SalI
SalI
pIBR4-
BamHI
(22Kb)
pBR322
SalI
SalI
BglII
NheI
SalI
pIBR6,6
SalI
(10.9Kb)
SalI
BHV-1 ST
genomic DNA
PCR
pVR1012
NheI-BglII
SalI-BamHI
SalI-NheI
7497
237
HindIII
CMV IE
promoter/activator
920
6574
kanamycin
resistance
6273
CMV IE
5' UT
1863
SalI
pPB156
(7691 bp)
NheI
StuI
StuI
5258
BGH
Term
BHV-1
gB
HinfI
4711
BglII

Figure No. 3

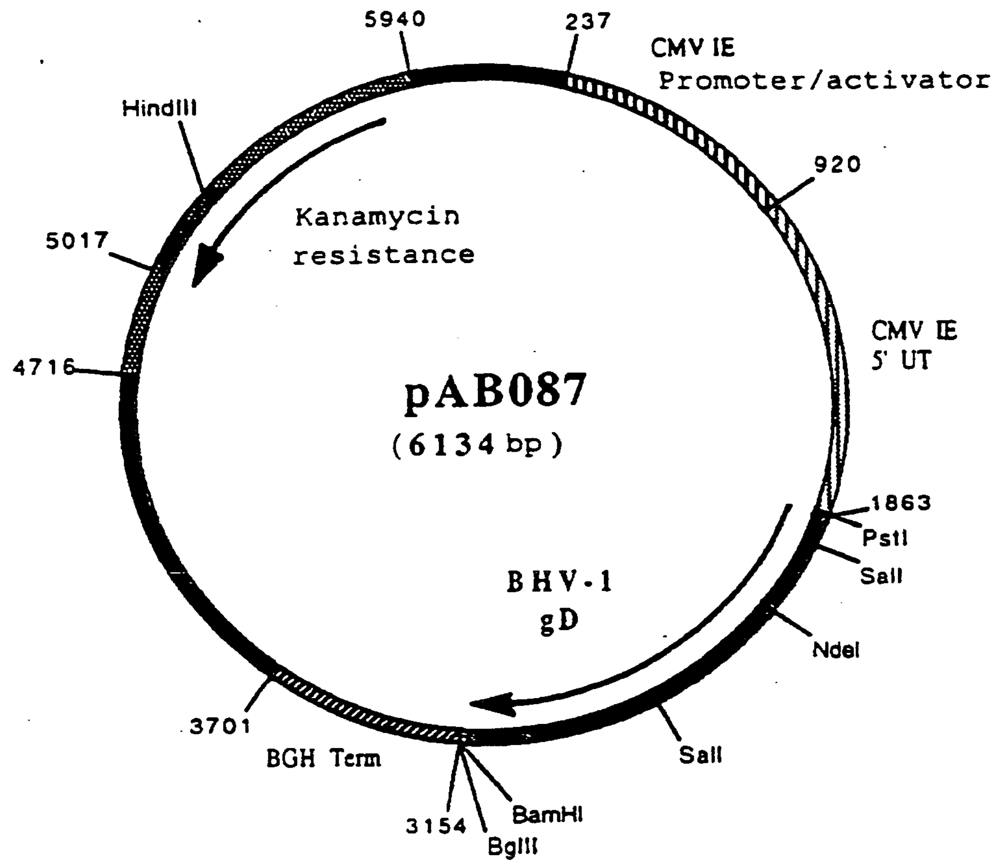

5940
237
CMV IE
Promoter/activator
HindIII
920
Kanamycin
resistance
5017
CMV IE
5' UT
4716
pAB087
(6134 bp)
1863
PstI
SalI
BHV-1
gD
NdeI
3701
SalI
BGH Term
BamHI
3154
BglII

Figure No. 4

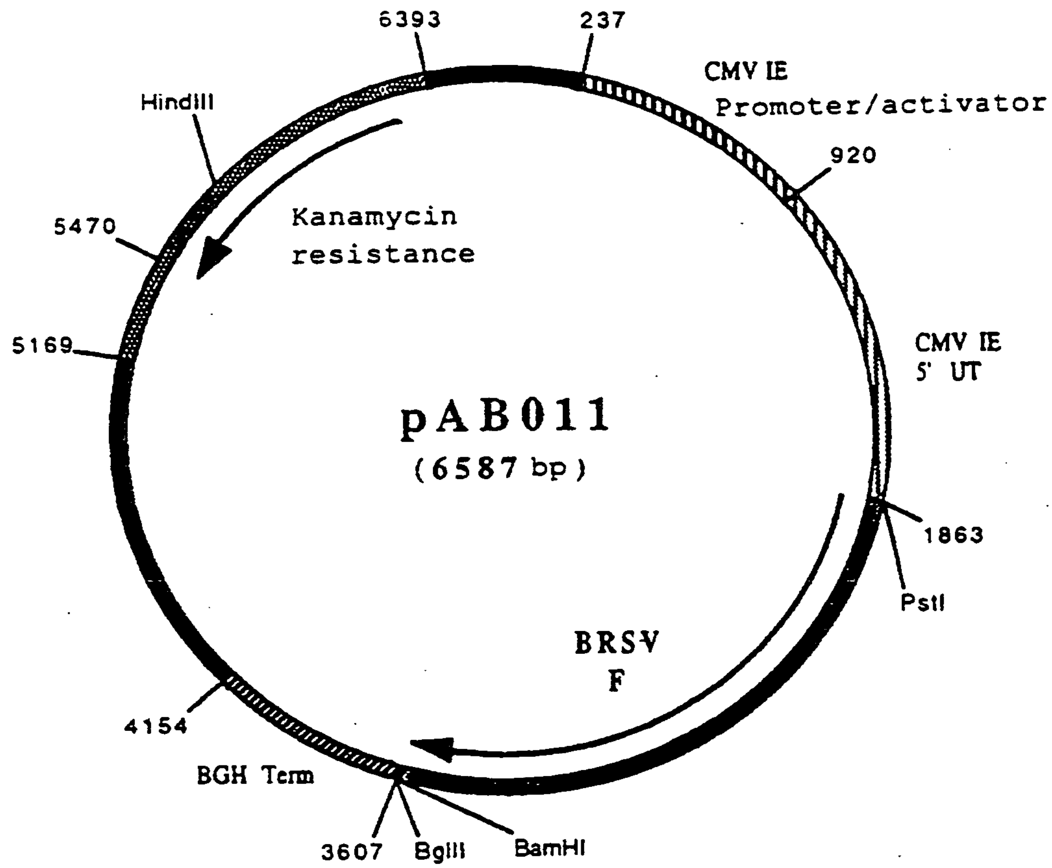

6393
237
CMV IE
Promoter/activator
920
HindIII
Kanamycin
resistance
5470
5169
CMV IE
5' UT
pAB011
(6587 bp)
1863
PstI
BRSV
F
4154
BGH Term
3607
BglII
BamHI

Figure No. 5

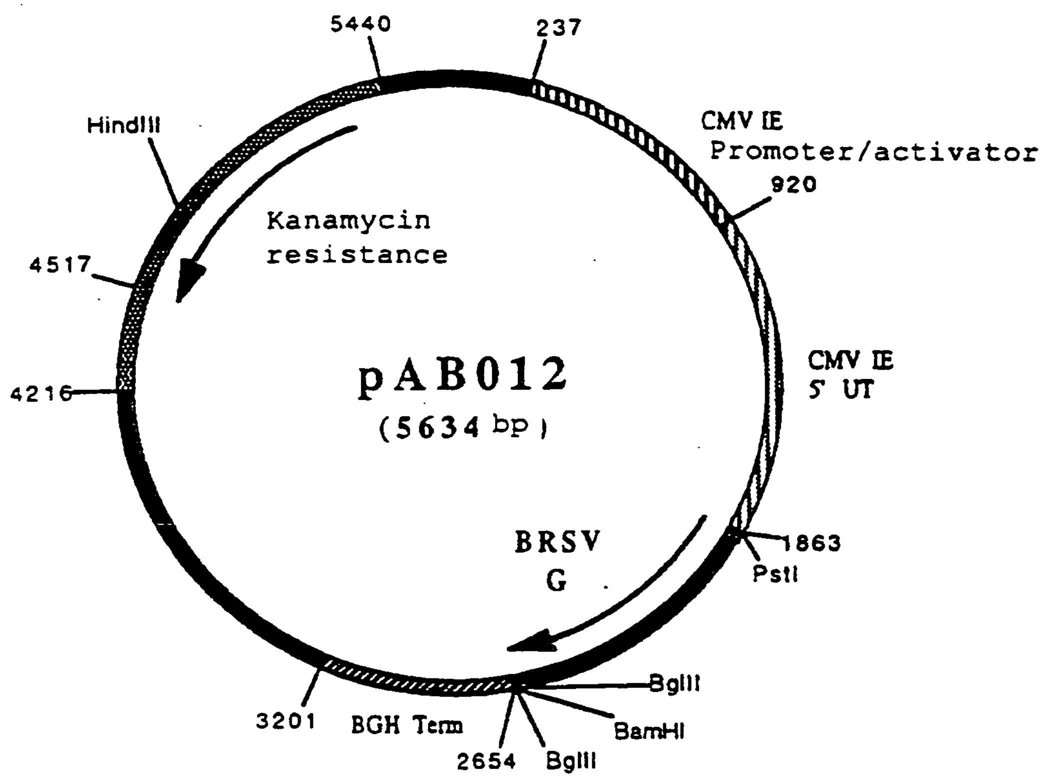

5440
237
HindIII
CMV IE
Promoter/activator
920
Kanamycin
resistance
4517
pAB012
(5634 bp)
CMV IE
5' UT
4216
BRSV
G
1863
PstI
BglII
BamHI
3201
BGH Term
2654
BglII

Figure No. 6

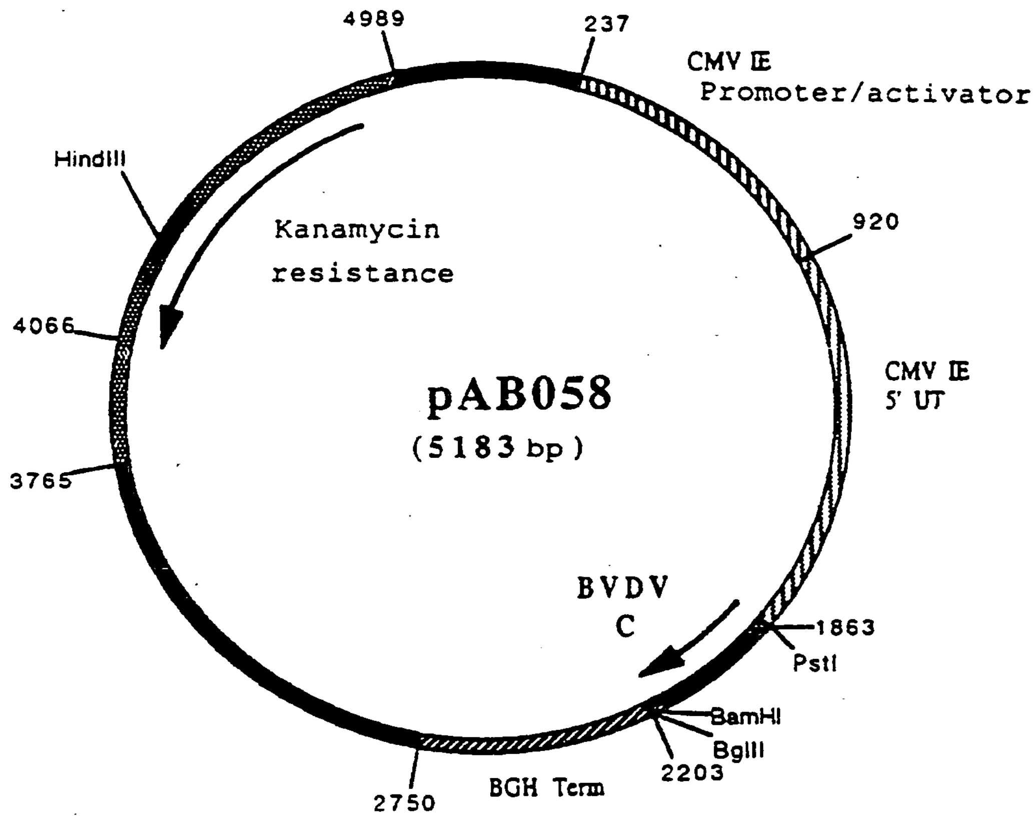

4989
237
CMV IE
Promoter/activator
HindIII
Kanamycin
resistance
920
4066
pAB058
(5183 bp)
CMV IE
5' UT
3765
BVDV
C
1863
PstI
BamHI
BglII
2203
BGH Term
2750

Figure No. 7

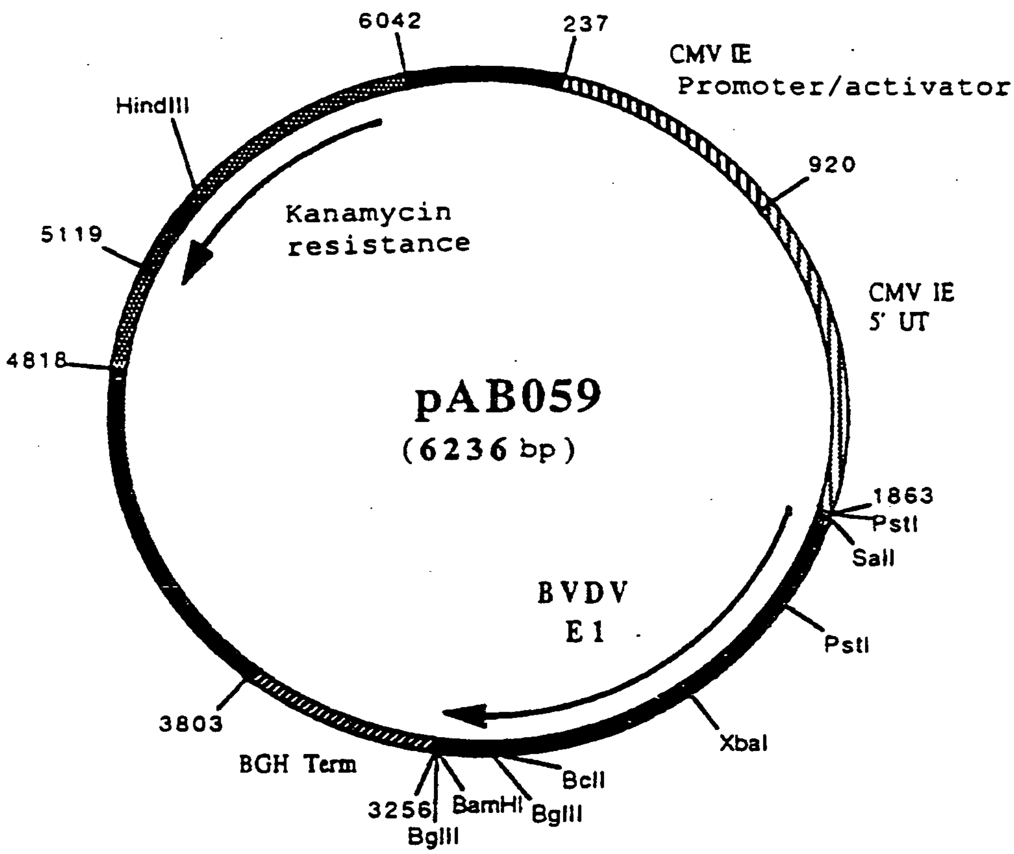
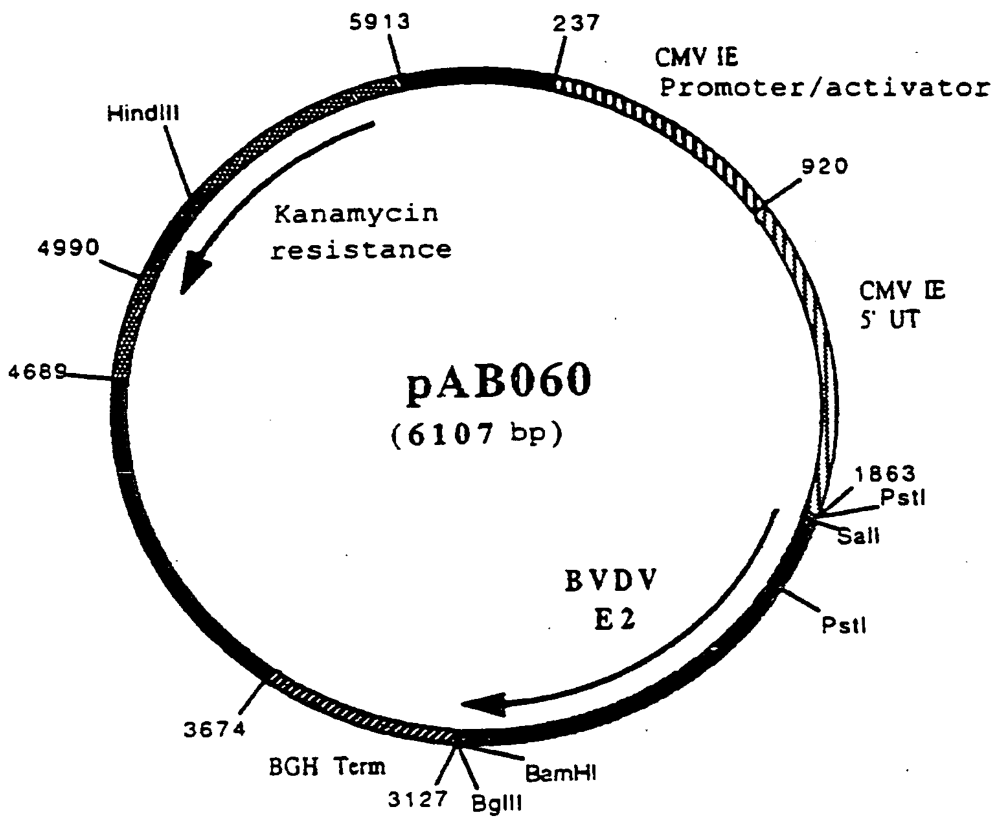

6042
237
CMV IE
Promoter/activator
HindIII
920
Kanamycin
resistance
5119
CMV IE
5' UT
4818
pAB059
(6236 bp)
1863
PstI
SalI
BVDV
E1
PstI
XbaI
3803
BGH Term
BclI
BamHI
BglII
3256
BglII

Figure No. 8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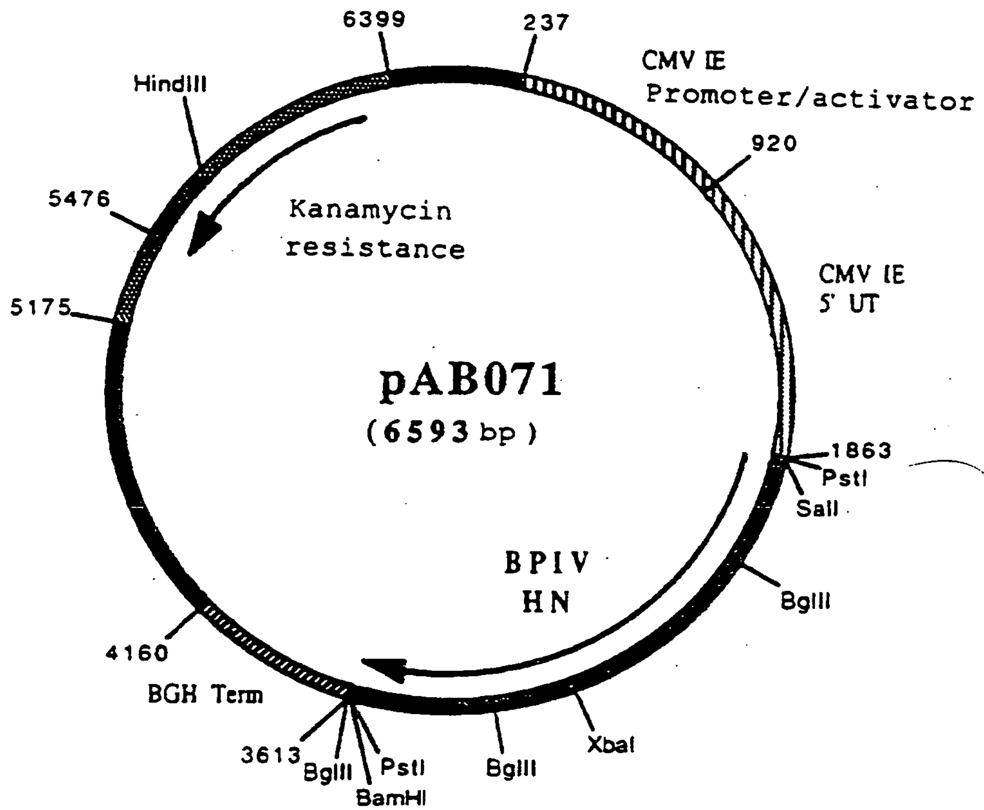
237
CMV IE
Promoter/activator
HindIII
920
Kanamycin
resistance
4990
CMV IE
5' UT
4689
pAB060
(6107 bp)
1863
PstI
SalI
BVDV
E2
PstI
3674
BGH Term
BamHI
3127
BglII

Figure No. 9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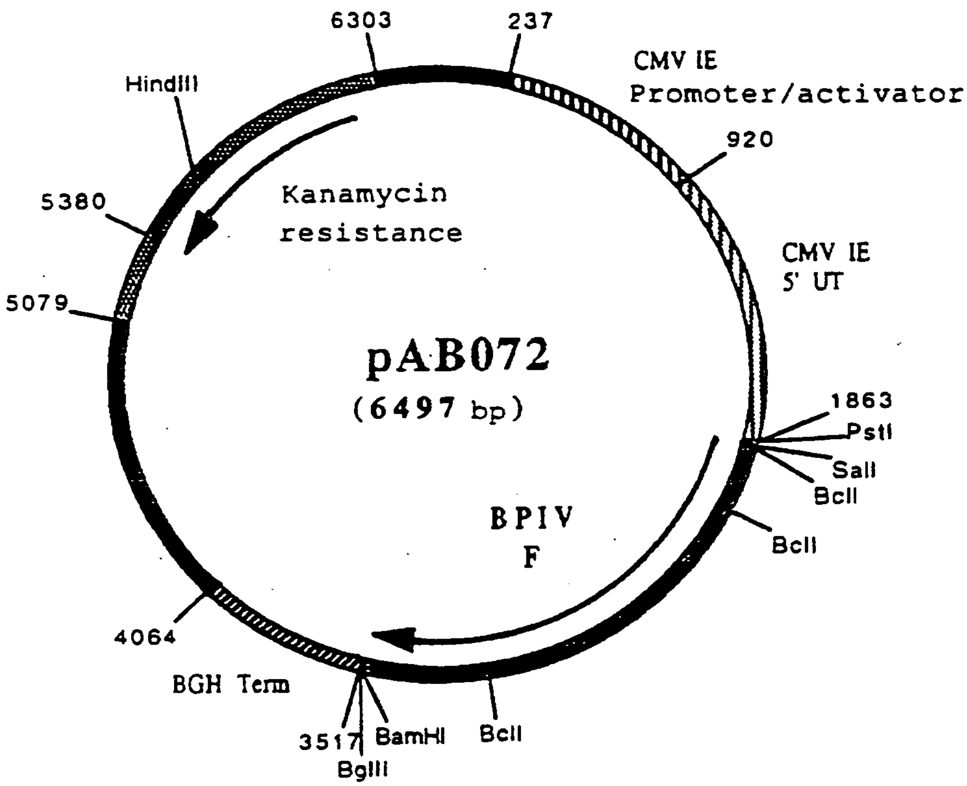
237
CMV IE
Promoter/activator
920
HindIII
5476
Kanamycin
resistance
5175
CMV IE
5' UT
pAB071
(6593 bp)
1863
PstI
SalI
BPIV
HN
BglII
4160
BGH Term
3613
BglII
PstI
BamHI
BglII
XbaI

Figure No. 10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237
CMV IE
Promoter/activator
HindIII
920
Kanamycin
resistance
5380
CMV IE
5' UT
5079
pAB072
(6497 bp)
1863
PstI
SalI
BclI
BPIV
F
BclI
4064
BGH Term
3517
BamHI
BclI
BglII

Figure No. 11